Practitioner's Docket No. 6360

CHAPTER II

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.' " M.P.E.P., § 601, 7th ed.

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/IT00/00048	16 February 2000	22 February 1999
INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

ISOLATION OF A N. CRASSA SILENCING GENE AND USES THEREOF
TITLE OF INVENTION

APPLICANT(S)
MACINO, Guiseppe and COGONI, Carlo

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231

ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. § 1.10*
*(Express Mail label number is **mandatory**.)*
(Express Mail certification is optional.)

I hereby certify that this Transmittal Letter and the papers indicated as being transmitted therewith is being deposited with the United States Postal Service on this date 8/20/01, in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL662368689US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Sarah E. Kennedy
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

NOTE: To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.

***WARNING:** Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing—See 37 C.F.R. § 1.8.*

NOTE: Documents and fees must be clearly identified as a submission to enter the national state under 35 U.S.C. § 371 otherwise the submission will be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).

I. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. § 371:

a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).

b. ☐ The U.S. National Fee (35 U.S.C. § 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
☒*	TOTAL CLAIMS	23 -20=	3	× $18.00=	$ 54.00
	INDEPENDENT CLAIMS	2 -3=	0	× $80.00=	$0.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	
BASIC FEE**	☐ U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: ☐ and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(1) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 C.F.R. § 1.492(a)(4)) ..... $100.00 ☐ and the above requirements are not met (37 C.F.R. § 1.492(a)(1)) ..... $690.00 ☒ U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: ☐ has been paid (37 C.F.R. § 1.492(a)(2)) ..... $710.00 ☐ has not been paid (37 C.F.R. § 1.492(a)(3)) ..... $1000.00 ☒ where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 C.F.R. § 1.492(a)(5)) $860.00				$860.00
	Total of above Calculations				= $914.00
SMALL ENTITY	Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (note 37 C.F.R. § 1.9, 1.27, 1.28)				-
	Subtotal				$914.00
	Total National Fee				$914.00
	Fee for recording the enclosed assignment document $40.00 (37 C.F.R. § 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				
TOTAL	Total Fees enclosed				$914.00

*See attached Preliminary Amendment Reducing the Number of Claims.

☒ Attached is a ☒ check ☐ money order in the amount of $ 914.00

☒ Authorization is hereby made to charge the amount of $ _____

☒ to Deposit Account No. 19-0079

☐ to Credit card as shown on the attached credit card information authorization form PTO-2038.

WARNING: *Credit card information should* ***not*** *be included on this form as it may become public.*

☒ Charge any additional fees required by this paper or credit any overpayment in the manner authorized above.

A duplicate of this paper is attached.

"WARNING: *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. ☒ A copy of the International application as filed (35 U.S.C. § 371(c)(2)):

NOTE: Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment. "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.

a. ☒ is transmitted herewith.

b. ☐ is not required, as the application was filed with the United States Receiving Office.

c. ☐ has been transmitted

i. ☐ by the International Bureau.
Date of mailing of the application (from form PCT/1B/308):
_____.

ii. ☐ by applicant on _____. *(Date)*

4. ☒ A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):

a. ☐ is transmitted herewith.

b. ☒ is not required as the application was filed in English.

c. ☐ was previously transmitted by applicant on _____. *(Date)*

d. ☐ will follow.

5. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

NOTE: *The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.*

a. ☐ are transmitted herewith.

b. ☐ have been transmitted

i. ☐ by the International Bureau.
Date of mailing of the amendment (from form PCT/1B/308):
_____.

ii. ☐ by applicant on _____. *(Date)*

c. ☒ have not been transmitted as

i. ☒ applicant chose not to make amendments under PCT Article 19.
Date of mailing of Search Report (from form PCT/ISA/210.):
7 September 2000

ii. ☐ the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. ☒ A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. § 371(c)(3)):

a. ☐ is transmitted herewith.

b. ☐ is not required as the amendments were made in the English language.

c. ☒ has not been transmitted for reasons indicated at point 5(c) above.

7. ☒ A copy of the international examination report (PCT/IPEA/409)

☒ is transmitted herewith.

☐ is not required as the application was filed with the United States Receiving Office.

8. ☐ Annex(es) to the international preliminary examination report

a. ☐ is/are transmitted herewith.

b. ☐ is/are not required as the application was filed with the United States Receiving Office.

9. ☐ A translation of the annexes to the international preliminary examination report

a. ☐ is transmitted herewith.

b. ☐ is not required as the annexes are in the English language.

10. ☒ An oath or declaration of the inventor (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115

a. ☐ was previously submitted by applicant on _____. Date

b. ☐ is submitted herewith, and such oath or declaration

i. ☐ is attached to the application.

ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. § 1.70.

c. ☒ will follow.

II. Other document(s) or information included:

11. ☒ An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):

a. ☒ is transmitted herewith.

b. ☐ has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _____.

c. ☐ is not required, as the application was searched by the United States International Searching Authority.

d. ☐ will be transmitted promptly upon request.

e. ☐ has been submitted by applicant on _____. Date

12. ☒ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:

a. ☒ is transmitted herewith.

Also transmitted herewith is/are:

☒ Form PTO-1449 (PTO/SB/08A and 08B).

☒ Copies of citations listed.

b. ☐ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).

c. ☐ was previously submitted by applicant on _____. Date

13. ☐ An assignment document is transmitted herewith for recording.

A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

_____

_____

_____

14. ☒ Additional documents:

a. ☒ Copy of request (PCT/RO/101)

b. ☒ International Publication No. WO 00/50581

i. ☒ Specification, claims and drawing

ii. ☐ Front page only

c. ☒ Preliminary amendment (37 C.F.R. § 1.121)

d. ☒ Other

Form PCT/IB/304; Form PCT/IB/308; Form PCT/IPEA/402; Form PCT/IB/332; Computer Readable Sequence Listing Disk and Statement

15. ☒ The above checked items are being transmitted

a. ☒ before 30 months from any claimed priority date.

b. ☐ after 30 months.

16. ☐ Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on _____, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependant claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ Please charge, in the manner authorized above, the following additional fees that may be required by this paper and during the entire pendency of this application:

☒ 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

☒ 37 C.F.R. § 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

☒ 37 C.F.R. § 1.17 (application processing fees)

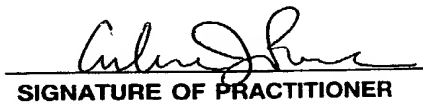
☒ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . . issue fee." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

☐ 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).



SIGNATURE OF PRACTITIONER

Arlene J. Powers

(type or print name of practitioner)

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Customer No.:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	Macino et al.	**GROUP:**	Unknown
SERIAL NO:	Unknown	**EXAMINER:**	Unknown
FILED:	Herewith		
FOR:	ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF		

Assistant Commissioner of Patents
Washington, D.C. 20231
Sir:

PRELIMINARY AMENDMENT

Preliminary to examination, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend the claims as follows:

7. (Amended) Expression vector comprising, under the control of a promoter that is expressed in bacteria, the nucleotide sequence according to [any one of claims 1-6] claim 1.

8. (Amended) Expression vector comprising, under the control of a promoter that is expressed in plant organs the nucleotide sequence according to [any one of claims 1-6] claim 1 in a sense and anti-sense orientation.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited on the date shown below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL662368689US addressed to the: Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Sarah Kennedy

Date: 8/20/01

9. (Amended) Expression vector comprising, under the control of a promoter that is expressed in fungi, the nucleotide sequence according to [any one of claims 1-6] claim 1 in a sense and anti-sense orientation.

10. (Amended) Expression vector comprising, under the control of a promoter that is expressed in animals, the nucleotide sequence according to [any one of claims 1-6] claim 1 in a sense and anti-sense orientation.

13. (Amended) Plant mutated at the nucleotide sequence according to [any one of claims 1-6] claim 1 having a reduced or inhibited silencing activity.

15. (Amended) Fungus mutated at the nucleotide sequence according to [any one of claims 1-6] claim 1 having a reduced or inhibited silencing activity.

17. (Amended) Non-human animal mutated at the nucleotide sequence according to [any one of claims 1-6] claim 1 having a reduced or inhibited silencing activity.

23. (Amended) Use of the nucleotide sequence according to [any one of claims 1-6] claim 1 to modulate the gene silencing in plants, animals and fungi.

REMARKS

The present preliminary amendment is submitted in order to correct the improper multiple dependency of claims as originally filed. Enclosed are clean copies of the claims.

Examination on the merits is respectfully requested.

Respectfully submitted,

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Samuels, Gauthier & Stevens
225 Franklin Street, Suite 3300
Boston, Massachusetts 02110
Telephone: (617) 426-9180
Extension 110

CLAIMS

1. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

2. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 1, wherein the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

3. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 2, wherein the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

4. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 3, wherein the domain is the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

5. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 4, wherein said nucleotide sequence encodes for a protein having the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

13. (Amended) Plant mutated at the nucleotide sequence according to claim 1 having a reduced or inhibited silencing activity.

14. Fungus transformed by using the expression vector active in fungi according to claim 9.

15. (Amended) Fungus mutated at the nucleotide sequence according to claim 1 having a reduced or inhibited silencing activity.

16. Non-human animal transformed by using the expression vector active in animals according to claim 10.

17. (Amended) Non-human animal mutated at the nucleotide sequence according to claim 1 having a reduced or inhibited silencing activity.

18. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

19. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 18, wherein the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

20. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 19, wherein the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

21. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 20, wherein the domain is the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

22. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 21 comprising the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

23. (Amended) Use of the nucleotide sequence according to claim 1 to modulate the gene silencing in plants, animals and fungi.

21. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 20, wherein the domain is the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

22. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 21 comprising the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

23. (Amended) Use of the nucleotide sequence according to claim 1 to modulate the gene silencing in plants, animals and fungi.

ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF

The present invention relates to the isolation and characterization of a *Neurospora crassa* gene encoding for an essential activity in the co-suppression process and to uses and applications thereof in vegetal, animal and fungine fields.

The production of transgenic organisms is of large utility both in basic and applied biological research. The transgenic DNA is usually integrated in the genome and transferred as a Mendelian character. However, in various instances, the transgene introduction induces gene silencing phenomena (Flavell, R.B. 1994), i.e. the repression of the expression of the transgene itself and/or of one or more endogenous homologous genes.

The gene silencing can act at two levels: transcriptional (trans-inactivation) where transgenes contain sequences homologous to the silenced gene promoter (Vaucheret, 1993); and post-transcriptional (co-suppression) which requires homologies between coding regions (Flavell, 1994; Stam et al., 1997; Baulcombe, 1996).

Generally the silencing induced by a transgene requires an almost complete sequence homology (from 70% to 100%) between transgene and silenced gene sequences (Elkind, 1990).

In the *Neurospora crassa* filamentous fungus, during the vegetative phase, the presence of transgenes induces a post-transcriptional gene silencing phenomenon, named "quelling" (Cogoni et al., 1996).

By using the *al-1* gene (albino 1) (Schmidhauser et al., 1990) as silencing visual marker, many features of the phenomenon have been discovered (Cogoni et al., 1996). Particularly the *al-1* gene "quelling" in *Neurospora* is characterized in that: 1) the gene silencing is reversible further to the loss of transgene copies; 2) the reduction of mRNA basal level results from a post-transcriptional effect; 3) transgenes containing at least a region of 132 base pairs which is identical to the region encoding for the target gene are sufficient to induce the "quelling"; 4) the duplication of promoter sequences is ineffective to induce the silencing; 5) the "quelling" exhibits a dominant behavior in eterocarions containing both transgenic and untransformed nuclei, indicating the involvement of a molecule which is acts "in trans" among the nuclei; 6) the expression of an aberrant RNA transcribed by the transgenic locus is strictly correlated to silencing, suggesting that the "quelling" can be induced and/or mediated by a transgenic RNA molecule.

Therefore homologies between *Neurospora* silencing and plant co-suppression can be pointed out. The gene silencing in *Neurospora* is reversible, as result of transgenic copies instability during mitotic phase; in plants also the co-suppression reversion is associated with the reduction of transgene copy number, resulting from intra-chromosomal recombination during mitosis or meiosis (Mittelstein Scheid et al., 1994; Stam et al., 1997). Thus both in plants and in *Neurospora* the transgene presence is required to maintain the silencing. As in *Neurospora,* a decrease of the mRNA basal level of the silenced gene results from a post-transcriptional

mechanism (Dehio and Schell 1994; van Blokand et al., 1994; de Carvalho et al., 1995). Furthermore to induce the "quelling", transgenes must contain a portion of the silencing target gene coding sequence, being the promoter region ineffective. In plants coding regions with no promoter sequences can induce silencing (van Blokand et al., 1994) and, as in the "quelling", promoters or functionally active gene products are not required for the co-suppression.

One of the similarities between "quelling" and co-suppression in plants is that both mechanisms are mediated by diffusion factors. In *Neurospora* eterokaryotic strains, nuclei wherein the *albino-1* gene is silenced are able to induce the *al-1* gene silencing of the other not transformed nuclei, all sharing the same cytoplasmic environment (Cogoni et al., 1996). In plants the presence of a diffusion factor results from the fact that the co-suppression is effective in inhibiting the replication of Tobacco Etch Virus (TEV), a RNA virus with an exclusively cytoplasmic cycle. The occurrence of highly diffusible factors, which are effective to mediate the co-suppression, has been demonstrated using the grafting technique in tobacco (Palaqui et al., 1997), showing that silenced tobacco plants are able to transfer the silencing to non-silenced plants through grafting.

The fact that "quelling" and co-suppression share all these features suggests that mechanisms involved in post-transcriptional gene silencing in plants and in fungi can be evolved by an ancestral common mechanism.

Recently gene inactivation phenomena resulting from transgene introduction have been disclosed in animals. In *Drosophila melanogaster* the location of a transgene close

to heterochromatic centers results in a variegate expression (Wallrath and Elgin, 1995; Pirrotta, V., 1997). Similar expression profiles have been observed when the reference transgene is within tandem arrayed transposons, indicating that tandem repeats are effective to induce the chromatin condensation. (Dorer and Henikoff, 1994). Again in *Drosophila* Pal-Bhadra et al. (1997) have observed that the transgene introduction can lead to gene inactivation phenomena, similar to the co-suppression.

Gene silencing phenomena resulting from transegene sequence repeats have been disclosed recently in mammalians.

Garrick et al. (1998) produced mouse transgenic lines wherein 100 transgenic copies are present only in a locus and are directly tandem arrayed. The transgene expression has been disclosed to be inversely proportional to the number of occurring copies, indicating that silencing phenomena dependent on repeat copies are present also in mammalians.

Therefore the identification of *Neurospora* genes which are involved in the silencing is the first step to modulate the same process in plants, animals and fungi. The silencing modulation is of great relevance when transgenic organisms able to express the desired phenotype are produced.

The authors of the present invention have already isolated *Neurospora crassa* strains having mutations regarding essential functions for gene silencing mechanism (Cogoni and Macino, 1997); 15 independent isolated mutants define three complementation groups, thus identifying the *qde-1, qde-2 and qde-3* genes (*qde*

stands for "quelling"-deficient), whose products are essential to the silencing machinery. *qde* genes are essential to the *Neurospora* silencing, as suggested by the fact that silencing of three independent genes (*al-1*, *al-2* and *qa-2*) is impaired by *qde* mutations (Cogoni and Macino, 1997).

The authors of the invention have identified and cloned now one out of *Neurospora qde* genes, the *qde-1* gene, thus identifying one of required factors for silencing. By considering the similarity between "quelling" and co-suppression, genes orthologous to the isolated gene are involved in co-suppression and more generally in gene silencing in other organisms, like plants, fungi and animals.

The present invention can be applied with reference to two general scope: 1) silencing potentiation as a tool for inactivating more effectively and durably a desired gene, and 2) silencing suppression to obtain a better expression of the introduced transgenes.

As to the silencing potentiation, the over-expression of one or more genes controlling the phenomenon can lead to higher efficiency and/or stability thereof. Therefore the introduction of *qde-1* gene or of homologous genes thereof in microorganisms can constitute a tool to repress more effectively gene functions. Particularly this approach is specially useful in plants wherein the co-suppression is usually used for the "knock-out" of gene functions. In plants again the gene silencing potentiation can be used to obtain lines resistant to pathogen virus, by introducing transgenes encoding for viral sequences, in order to achieve the

expression inhibition of the virus itself (Flavell et al., 1994).

Analogous applications are suitable for animals, wherein some indications suggest that silencing can inhibit the suitable expression of introduced transgenes (Garrick et al., 1998).

On the contrary, there are instances wherein it is desirable not to have or to reduce the gene silencing, i.e. where a transgene is to be over-expressed. It is known that the co-suppression is strictly correlated both with the presence of an high copy number of the transgene, and with a transgene high expression. This correlation can hamper the production of transgenic organisms which express a transgene at high levels, because more high is the expression and/or the copy number, more probable is to evoke silencing responses. As above mentioned, analogous mechanisms of gene inactivation, dependent on a high copy number, have been disclosed in animals. In these circumstances plant or animal lines, totally or partially ineffective for silencing, constitute an ideal recipient wherein the desired gene can be over-expressed. The invention can be applied within this scope using different approaches:

A) Identification and production of mutant lines in genes homologous to *qde-1* gene, in plants, animals and fungi.

The knowledge of *Neurospora qde-1* gene, essential for silencing mechanism, can allow the isolation of mutant lines in other organisms, mutated in genes homologous to *qde-1*. For example by means of amplifications using degenerated primers, designed from the most conserved regions of *qde-1* gene, mutant lines in

homologous genes can be identified, by analysis of insertion mutant gene banks, already available for many plant species. Both in fungi and animals such mutants can be obtained, following the identification of the homologous gene, by means of "gene disruption" techniques using homologous recombination.

B) Reduction of *qde-1* gene expression

Other strategies for the production of silencing-deficient lines comprise the use of *Neurospora qde-1* gene or homologous genes thereof. *qde-1* or homologous genes can be introduced into suitable expression vectors to express them in an anti-sense orientation in order to inhibit the expression of resident endogenous genes. Alternatively portions of *qde-1* or of homologous genes can be over-expressed, in order to obtain a negative dominant effect and thus blocking the function of *qde-1* endogenous genes.

The authors of the present invention have cloned and characterised the *Neurospora crassa qde-1 gene*. The sequence analysis of the *qde-1* gene detected a region having a significant homology with a RNA-dependent RNA polymerase, isolated from tomato, which was suggested, but not demonstrated, to be involved in the co-suppression mechanism (Schiebel et al., 1998).

The authors of the invention for the first time have demonstrated that a gene encoding for a RNA-dependent RNA polymerase is involved in gene silencing induced by transgenes. Therefore for the first time it is disclosed that a gene belonging to the RNA-dependent RNA polymerase family is an essential component also for inactivation mechanism of the repeat sequences.

Within the scope of the invention the reference to homology per cent means similarity per cent, i.e. number of identical residues + number of conserved residues with respect to the total residues of the considered sequence.

Therefore it an object of the present invention a nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA dependent RNA polymerase domain, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Preferably the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. More preferably the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Most preferably the domain comprises the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. According to a particular embodiment the nucleotide sequence encodes for a protein having the amino acid sequence of SEQ ID No. 1 or functional portions thereof. Even more preferably the nucleotide sequence is the nucleotide sequence of SEQ ID No. 1 or its complementary sequence.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in bacteria, the nucleotide sequence of the invention. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the expression in bacteria can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in plants or in specific plant organs, the

nucleotide sequence of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the invention in plants or in specific plant organs can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in fungi, the nucleotide sequence of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the inventive protein in fungi can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in animals, the nucleotide sequence of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the invention in animals can be used and it is within the scope of the invention.

A further object of the invention is a prokaryotic organism transformed by using the expression vector active in bacteria of the invention.

A further object of the invention is a plant or a specific plant organ transformed by using the expression vector active in plants of the invention.

A further object of the invention is a plant mutated at the nucleotide sequence of the invention having a reduced or inhibited silencing activity.

A further object of the invention is a fungus transformed with the expression vector of the invention active in fungi.

A further object of the invention is a fungus mutated at the nucleotide sequence of the invention and having reduced or inhibited silencing activity.

A further object of the invention is a non-human animal transformed with the expression vector of the invention active in animals.

A further object of the invention is a non-human animal mutated at the nucleotide sequence of the invention and having a reduced or inhibited silencing activity.

A further object of the invention is a not human animal mutated at the nucleotide sequence of the invention and having reduced or inhibited silencing activity.

A further object of the invention refers to a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polimerase domain, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Preferably the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. More preferably the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Most preferably the domain comprises the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. According to a particular embodiment the nucleotide sequence encodes for a protein having the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

It is within the scope of the present invention the use of the nucleotide sequence of the invention to modulate gene silencing in plants, animals and fungi.

The present invention now will be disclosed by way of non limiting examples with reference to the following figures:

Figure 1 shows the restoration of the *al-1* expression in 107 insertional mutant strain. The total RNA has been extracted from mycetes collected after light induction over ten minutes from an *al-1* silenced strain (6XW), a untransformed wild type strain (WT) and 107 mutant strain. For the hybridization an *al-1* specific probe was used. In the lower part the restoration using an *al-1* specific probe is showed.

Figure 2 shows the genomic organization of the *qde-1* gene. a) The two cosmides (56G11 and 40H7) able to complement the *qde-1* mutants are represented. The white box in the 40H7 cosmid represents the sequences of the cosmid vector. A restriction map of 7,9 Kb *qde-1* containing fragment obtained from 40H7 using EcoRi is showed: E(EcoRI), P(PstI), B(BgIII). The black box represents the ORF identified within EcoRi 7,9 Kb fragment. The pDX and pSX plasmids containing the DNA fragments subcloned in the XbaI (X) and EcoRI (E) sites are also showed. B) Southern analysis of the 107 and WT strains. The genomic DNA was digested using BgII and NaeI. In the lower diagram the DNA probe used for the hybridization and the expected BgII/NaeI(B/N) restriction fragments are reported. The triangle represents the integration site in the 107 strain which determines the disappearance of the 1,0 Kb restriction fragment.

Figure 3 represents the expression of the *qde-1* gene in the 107 insertional mutant strain, untransformed wild type (WT) strain and al-1 silenced strain (6XW). The total RNA was hybridizated using a *qde-1* specific probe. In the lower part the amount of gel loaded RNA is showed.

Figure 4 represents the amino acid sequence deduced from the *qde-1* gene. The underlining indicates the RdRP conserved domain as showed in the alignment of Figure 5.

Figure 5 represents a sequence alignment of the QDE-1 protein with other polypeptides from SwissProtein sequence database: ORF from Z488334 (eleg1) C. *elegans*, ORF from Z98533 (pom) *S. pombe*, ORF from AF080120 (araB) *A. thaliana* and RNA-dependent RNA polymerase from Y104403 (RdRP) tomato. Identical residues are pointed out in black, whereas the conservative replacements are showed in gray.

Materials and Methods

Strains, growing and transforming conditions

The methodology and heterokaryon analysis in *Neurospora crassa* substantially was the same as described in (Davis and De Sevres, 1970). The spheroplasts are prepared according to method of Vollmer and Yasnofsky (1997). The 107 strain was isolated in the following way: a *qde-1* silenced strain, called 6xw, already described (Cogoni and Macino, 1997), was transformed with pMXY2 which contains the benomyl resistant beta-tubulin gene, which acts as dominant selectable marker in *N. Crassa* (Staben et al. 1989). Transformed strains able to grow in the presence of benilate containing medium were selected on the base of the carotenoid biosynthesis by visualization of the conidium colors: the conidia from the wild type strains were bright orange, whereas those

from transformed strains having colors from white to yellow were indicative of a silencing activity.

Plasmids and gene libraries

The genomic gene *qde-1* was isolated from a *N. Crassa* gene library in cosmides (Cabibbo et al., 1991). The sub-cloning of the restriction fragments from the gene library clones was carried out in the pBSK plasmid. Therefore the sub-clones were used in co-transforming experiments using pMXY2 or pES200 (containing the hygromycin resistant gene).

Southern and Northern Hybridizations

Chromosomal DNA was prepared according to Morelli et al. (1993). After digestion, the genomic DNA was transferred according to Maniatis et al. (1982). The probes were labeled by casual priming (Boheringer). The RNA was electrophoresed on agarose gel, transferred and blotted on Hybond N membranes.

DNA Analysis and Sequencing

The *qde-1* nucleotide sequence was determined for both strands using TAQ FS polymerase and the fluorescence method and analyzed using an Applied Biosystems 373A automated apparatus; the nucleotide and amino acid derived sequences were analyzed by means of MacMolly Tetra program. A protein comparison was carried out using the BLASTP method. The ClustaIW algorithm was used for the alignment.

Results

In order to clone the *qde* genes an insertional mutagenesis on an *al-1* transgenic strain (6XW) which shows an albino phenotype (white) resulting from a post-transcriptional silencing of the *al-1* endogenous gene was used: out of 100.000 independent transformed insertional

strains, a strain (107) showed a reversion of the gene silencing visible as restoration of a bright orange wild type phenotype. The bright orange wild type phenotype of the 107 strain results from the restoration of the expression of *al-1* mRNA, as demonstrated by a Northern analysis (see Figure 1). Furthermore an heterokaryon assay revealed the mutation to be recessive and *trans* acting. In addition by means of the heterokaryon assay it was possible to establish that the 107 strain mutant belongs to one of the three already identified complementation *qde* groups (Cogoni and Macino 1997). The restoration of an *al-1* silenced phenotype occurs in heterokaryons with *qde-2* and *qde-3* mutants. It is not possible to complement with *qde-1* mutants (Table 1), indicating that the 107 strain is mutated at the *qde-1* gene.

Table 1 - The 107 strain is mutated at the *qde-1* gene

qde mutant strains used in specific heterokaryons							
	107	M17	M18	M10	M11	M7	M20
107	WT	AL	AL	AL	AL	WT	WT
M17		WT	WT	AL	AL	AL	AL
M18			WT	AL	AL	AL	AL
M10				WT	WT	AL	AL
M11					WT	AL	AL
M7						WT	WT
M20							WT

WT = heterocaryon with a wild type phenotype for carotenoid (bright orange);

AL = heterocaryon with an albino phenotype wherein the *al-1* gene silencing is restored.

The *qde* mutant strains were described by Copgoni and Macino (1997); M17 and M18 are *qde-3* mutants; M10 and

M11 are *qde-2* mutants; M7 and M20 are *qde-1* mutants. The heterokaryon phenotypes were examined by visual inspection after a seven day growth in the presence of light.

In order to isolate the *qde-1 gene,* the "tagging" plasmid was recovered from the 107 strain by means of a procedure suitable for the liberation of the plasmid using the *BglII* restriction enzyme having a single restriction site within the tagging plasmid. The pCR4 plasmid was recovered after chromosomal DNA re-ligation following a *Bgl*II restriction (see figure 2a). The chromosomal DNA flanking the insertion site was isolated using the *BglII* and *PstI* enzymes and the resultant restriction fragment was used to probe a *N. crassa* genomic library in cosmids. Cosmids 58G11 and 40H7, both positively hybridizating, were introduced by means of an UV induced transforming experiment in the 107 strain and in the M7 qde-1 mutant strain. Both the cosmids were able to complement the two assayed *qde-1* mutants. The restoration of the *al-1* gene silencing which determines the appearance of a white phenotype indicates that both the cosmids contain a functional *qde-1* gene. By using the same flanking DNA like a probe, two EcoRi fragments from 40H7 and 58G11, respectively having 7,9 and 10,0 kB, were identified and subcloned (see figure 2a). Both the EcoRi fragments (p79E and p10E plasmids) are able to complement the *qde-1* strain (107 and M7) but not the *qde-2* (M10) and *qde-3* (M17) mutant strains (Table 2), indicating that the *qde-1* functional gene is contained in the 7,9 Kp EcoRi fragment.

Table 2 Complementation with the *qde-1* gene

Plasmids	qde mutant strains used			
	107	M7(qde-1$^-$)	M10(qde-2$^-$)	M17(qde-3$^-$)
P79E	58/200(29%)	48/200(24%)	0/200(0%)	0/200(0%)
P10E	51/100(25%)	25/100(25%)	0/200(0%)	0/200(0%)
PSX	0/200(0%)	0/200(0%)	-	-
PDX	0/200(0%)	0/200(0%)	0/200(0%)	0/200(0%)

The complementation frequency is reported as per cent of the transforming strains which show an albino phenotype with respect to the total number of transforming strains. The pMXY2 plasmid was used as negative control.

The ability of the *qde-1* gene in restoring the *al-1* gene silencing only in corresponding mutants, excludes the possibility that the DNA cloned fragment is able to restore the *al-1* gene silencing, apart from the *qde* complementation group. In addition the M7 strain transformed with the 7,9 Kb EcoRI fragment allows the ability in silencing an other *al-2* carotenogenic gene when introduced by transformation (not showed data). The 7,9 Kb EcoRI fragment was further cloned using the XbaI site (see figure 2a) which cuts the center the EcoRI fragment. Both the XbaI/EcoRI (pSX and pDX plasmids) fragments were not able to complement the 107 and M7 strains (see Table 2), suggesting that the XbaI site is probably localised within the *qde-1* gene.

The whole region of the EcoRI fragment which includes the putative *qde-1* gene and the adjacent regions were sequenced, revealing an open reading frame (ORF) of 4206 bp which encodes for a putative protein containing 1402 amino acids. Two different results suggest that this ORF corresponds to the *qde-1* gene. Firstly the XbaI

restriction site used to subclone the 7,9 Kb EcoRI fragment is localized in the center of the ORF (figure 2a) and therefore is consistent with the result that both the KbaI/EcoRI fragments are not able to complement the *qde-1* mutation (Table 2). Secondly by means of Southern analysis (see Figure 2b) the insertion site of the tagging plasmid in the 107 strain was mapped to be within the BglII and NaeI restriction sites in the ORF. No size variation of the flanking regions was detected, therefore excluding the possibility that the deletions include other ORFs within the 7,9 Kb EcoRi region. The cDNAs synthesized by inverted PCR (RT-PCR) revealed a co-linearity with the genomic DNA indicating that no intron is present. The expression of the *qde-1* gene was analyzed by Northern analysis using a probe including the *qde-1* ORF (figure 2a). Thus a transcript of about 5000 nt was detected and further it was found out that the *qde-1* mRNA basal level in an *al-1* silenced strain (6XW) was twice than in a not transformed WT strain (see figure 3). In addition the *qde-1* whole length mRNA is not detectable in the *qde-1* 107 insertional mutant strain where, on the contrary, a smaller band is included suggesting that the *qde-1* truncated transcripts are produced as the integration result. The fact that the *qde-1* gene expression is specifically increased in a silenced strain suggests the existence of a regulatory mechanism able to activate cell components of the silencing machinery in the transgenic strains.

The QDE-1 protein deduced from the nucleotide sequence contains 1402 amino acids (see figure 4), the molecular weight and statistical pI thereof being 158.004 Da and 8.0, respectively. The QDE-1 protein does not

contain a signal peptide or a transmembrane domain indicating that it is probably an intracellular protein. Furthermore the idiopathic plot suggests that QDE-1 is a soluble protein. A BLAST study showed that *qde-1* has an homology statistically significant with hypothetical proteins from various other organisms comprising: two ORFs from *C. elegans* (Z4834 and Z78419 EMBL entry numbers) with expected values (E value) of 2e-16 and 9e-10, respectively, one ORF from *S. pombe* (Z98553 EMBL entry number) with an 3e-13 E value; four ORFs from *A. thaliana* (AF080120 and AC005169 EMBL entry numbers, the latter comprising thre ORFs at the same chromosomal localization,) and 8e-15, 7e-06, 4e-05, 5e-02 E values, respectively. Finally a significant homology (2e-17 E value) with a putative protein coded by tomato cDNA (Y10403 EMBL entry number) was discovered. The discovered homology does not extend over the whole protein but it is limited to a portion containing 570 amino acids, from aa. 710 to aa. 1282, which defines a conserved domain (see figure 5). Among the identified putative homologous proteins only that derived from the sequence of tomato cDNA was functionally characterized as a RNA-dependent RNA polymerase (RdRP, 9).

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ttg gac aag gta ccg act cga agg cat gcc aat acg aga gat ccc acg   3223
Leu Asp Lys Val Pro Thr Arg Arg His Ala Asn Thr Arg Asp Pro Thr
    245                 250                 255

gcg aca ggt cat aga cga gcg gac cag gtg gat tcc ttt gat aca tct   3271
Ala Thr Gly His Arg Arg Ala Asp Gln Val Asp Ser Phe Asp Thr Ser
260                 265                 270                 275

caa ggc act tcc tat ggt tcg agt gtc ttc agc gct tgc cgt cac aat   3319
Gln Gly Thr Ser Tyr Gly Ser Ser Val Phe Ser Ala Cys Arg His Asn
                280                 285                 290

cag agc act acc cag agt agt ttt gag gct cct cct tca cag ccc aga   3367
Gln Ser Thr Thr Gln Ser Ser Phe Glu Ala Pro Pro Ser Gln Pro Arg
            295                 300                 305
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gag aag cgg cct gtg gat gcc acg gtc ttt gag gct gga cac ttg att   3415
Glu Lys Arg Pro Val Asp Ala Thr Val Phe Glu Ala Gly His Leu Ile
        310                 315                 320

gag tct cct agc aaa gga aga aca acc aag tcc cac ata gat aac cag   3463
Glu Ser Pro Ser Lys Gly Arg Thr Thr Lys Ser His Ile Asp Asn Gln
    325                 330                 335

ccc ctt tca tcg tct tcc cag ggt gaa act tcg ttc agc act tac tat   3511
Pro Leu Ser Ser Ser Ser Gln Gly Glu Thr Ser Phe Ser Thr Tyr Tyr
340                 345                 350                 355

gag tcg ttt cca agt tcc ggc ggc gag ggc gca att ccc gag ccg agt   3559
Glu Ser Phe Pro Ser Ser Gly Gly Glu Gly Ala Ile Pro Glu Pro Ser
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cgc tca aat gga ctg gct cgg agc gaa gaa agc gct cga tct cag gtt   3607
Arg Ser Asn Gly Leu Ala Arg Ser Glu Glu Ser Ala Arg Ser Gln Val
            375                 380                 385

caa gtt cat gcc ccg gtg gtt gca gct cgg ctg aga aat att tgg ccg   3655
Gln Val His Ala Pro Val Val Ala Ala Arg Leu Arg Asn Ile Trp Pro
        390                 395                 400

aaa ttt ccc aaa tgg cta cac gaa gct cct ctc gct gtt gca tgg gaa   3703
Lys Phe Pro Lys Trp Leu His Glu Ala Pro Leu Ala Val Ala Trp Glu
    405                 410                 415

gtt acc aga ctc ttt atg cac tgc aaa gta gac ttg gaa gac gag agc   3751
Val Thr Arg Leu Phe Met His Cys Lys Val Asp Leu Glu Asp Glu Ser
420                 425                 430                 435

ctg ggc cta aag tac gac cct tcc tgg tct acc gcg cgc gat gtc aca   3799
Leu Gly Leu Lys Tyr Asp Pro Ser Trp Ser Thr Ala Arg Asp Val Thr
                440                 445                 450
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gat atc tgg aag act ctc tac cgg ctt gat gct ttc cgt ggt aaa ccc   3847
Asp Ile Trp Lys Thr Leu Tyr Arg Leu Asp Ala Phe Arg Gly Lys Pro
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ttt cca gaa aag ccg ccc aac gac gtg ttc gtg acc gca atg acg ggc   3895
Phe Pro Glu Lys Pro Pro Asn Asp Val Phe Val Thr Ala Met Thr Gly
        470                 475                 480

aac ttt gag agc aaa ggt agt gcc gtt gtt ctc tct gct gtt cta gac   3943
Asn Phe Glu Ser Lys Gly Ser Ala Val Val Leu Ser Ala Val Leu Asp
    485                 490                 495

tac aat ccg gac aac tcg cct act gcg ccc ctt tac ctt gtg aag ctg   3991
Tyr Asn Pro Asp Asn Ser Pro Thr Ala Pro Leu Tyr Leu Val Lys Leu
500                 505                 510                 515

aag ccg ctc atg ttc gag cag ggc tgt cga ctc acc cgt cgg ttc ggt   4039
Lys Pro Leu Met Phe Glu Gln Gly Cys Arg Leu Thr Arg Arg Phe Gly
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cct gat agg ttt ttc gag atc ctt ata ccg tcg cct acg agc acc agc   4087
Pro Asp Arg Phe Phe Glu Ile Leu Ile Pro Ser Pro Thr Ser Thr Ser
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cca agt gta ccg ccg gtg gtc agc aaa caa cca ggt gcg gtc gaa gaa   4135
Pro Ser Val Pro Pro Val Val Ser Lys Gln Pro Gly Ala Val Glu Glu
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gtc atc cag tgg ctc acg atg ggg caa cat tct ctt gta ggc cgc caa   4183
Val Ile Gln Trp Leu Thr Met Gly Gln His Ser Leu Val Gly Arg Gln
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tgg cgc gct ttc ttc gcc aaa gat gcc gga tac agg aaa cct ctc agg   4231
Trp Arg Ala Phe Phe Ala Lys Asp Ala Gly Tyr Arg Lys Pro Leu Arg
580                 585                 590                 595
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gag ttc cag ctc cgc gcc gag gac ccg aaa ccc atc atc aag gag aga   4279
Glu Phe Gln Leu Arg Ala Glu Asp Pro Lys Pro Ile Ile Lys Glu Arg
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gtc cac ttc ttt gcc gag acc ggc att acg ttc cga cct gat gtg ttc   4327
Val His Phe Phe Ala Glu Thr Gly Ile Thr Phe Arg Pro Asp Val Phe
            615                 620                 625

aag acg aga tct gtc gtt ccg gca gag gaa cct gta gag caa cgg acc   4375
Lys Thr Arg Ser Val Val Pro Ala Glu Glu Pro Val Glu Gln Arg Thr
        630                 635                 640

gag ttc aaa gtt agt caa atg ctg gac tgg ctc ctg caa ctc gac aac   4423
Glu Phe Lys Val Ser Gln Met Leu Asp Trp Leu Leu Gln Leu Asp Asn
    645                 650                 655

aac act tgg cag ccg cac ctc aag ttg ttc tcc cgt atc cag ctc ggt   4471
Asn Thr Trp Gln Pro His Leu Lys Leu Phe Ser Arg Ile Gln Leu Gly
660                 665                 670                 675

ctg agt aag aca tat gcc att atg aca ttg gag cct cac cag atc aga   4519
Leu Ser Lys Thr Tyr Ala Ile Met Thr Leu Glu Pro His Gln Ile Arg
                680                 685                 690

cac cac aag acc gat ctt ctt tca cct tca ggc act ggc gaa gtg atg   4567
His His Lys Thr Asp Leu Leu Ser Pro Ser Gly Thr Gly Glu Val Met
            695                 700                 705

aat gac ggt gta ggc cgc atg tcg cga agc gtg gcc aag agg ata cgc   4615
Asn Asp Gly Val Gly Arg Met Ser Arg Ser Val Ala Lys Arg Ile Arg
        710                 715                 720

gat gtt ctc ggt ttg ggt gat gtg ccc tct gct gtg caa ggg cgg ttt   4663
Asp Val Leu Gly Leu Gly Asp Val Pro Ser Ala Val Gln Gly Arg Phe
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ggt tcg gcc aag gga atg tgg gtt atc gac gtt gac gac aca ggc gat   4711
Gly Ser Ala Lys Gly Met Trp Val Ile Asp Val Asp Asp Thr Gly Asp
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gag gat tgg atc gag aca tac ccg tcc cag cgc aag tgg gaa tgc gac   4759
Glu Asp Trp Ile Glu Thr Tyr Pro Ser Gln Arg Lys Trp Glu Cys Asp
                760                 765                 770

ttc gtt gat aaa cat caa cgt acc ctc gaa gtc cgg agc gtg gct tct   4807
Phe Val Asp Lys His Gln Arg Thr Leu Glu Val Arg Ser Val Ala Ser
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gaa ctg aag tca gct ggt ctc aac cta cag ctg tta cct gtc ctg gaa   4855
Glu Leu Lys Ser Ala Gly Leu Asn Leu Gln Leu Leu Pro Val Leu Glu
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Asp Arg Ala Arg Asp Lys Val Lys Met Arg Gln Ala Ile Gly Asp Arg
    805                 810                 815

ctt atc aac gat ttg caa cga cag ttc agc gag caa aag cat gct ttg   4951
Leu Ile Asn Asp Leu Gln Arg Gln Phe Ser Glu Gln Lys His Ala Leu
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aat cgc cca gtg gaa ttt cgc caa tgg gtt tac gag agt tat tcc agt   4999
Asn Arg Pro Val Glu Phe Arg Gln Trp Val Tyr Glu Ser Tyr Ser Ser
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cgc gca act cga gtc agc cac ggc cgt gtg cct ttt ctt gct ggg cta   5047
Arg Ala Thr Arg Val Ser His Gly Arg Val Pro Phe Leu Ala Gly Leu
            855                 860                 865

cct gac agt caa gag gag aca ctg aac ttc ttg atg aac agt ggg ttc   5095
Pro Asp Ser Gln Glu Glu Thr Leu Asn Phe Leu Met Asn Ser Gly Phe
        870                 875                 880
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gat ccc aag aag caa aag tac ttg caa gac atc gcc tgg gat ctt caa   5143
Asp Pro Lys Lys Gln Lys Tyr Leu Gln Asp Ile Ala Trp Asp Leu Gln
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aag cgg aaa tgt gac acg ttg aag tcc aag ctg aac atc cgt gtc ggt   5191
Lys Arg Lys Cys Asp Thr Leu Lys Ser Lys Leu Asn Ile Arg Val Gly
900                 905                 910                 915

cga tca gca tac att tac atg att gcc gat ttc tgg ggt gtg ctt gag   5239
Arg Ser Ala Tyr Ile Tyr Met Ile Ala Asp Phe Trp Gly Val Leu Glu
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gaa aat gag gtt cat gtc gga ttc tcc tca aag ttc aga gac gag gag   5287
Glu Asn Glu Val His Val Gly Phe Ser Ser Lys Phe Arg Asp Glu Glu
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gag tct ttt aca ctc cta tcg gac tgt gat gtc ctc gtg gcg cga tcc   5335
Glu Ser Phe Thr Leu Leu Ser Asp Cys Asp Val Leu Val Ala Arg Ser
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Pro Ala His Phe Pro Ser Asp Ile Gln Arg Val Arg Ala Val Phe Lys
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Pro Glu Leu His Ser Leu Lys Asp Val Ile Ile Phe Ser Thr Lys Gly
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gat gta ccg ctt gct aag aag cta tct ggt gga gac tac gac ggc gat   5479
Asp Val Pro Leu Ala Lys Lys Leu Ser Gly Gly Asp Tyr Asp Gly Asp
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atg gcc tgg gtc tgc tgg gat ccg gag atc gtc gat ggt ttc gtc aat   5527
Met Ala Trp Val Cys Trp Asp Pro Glu Ile Val Asp Gly Phe Val Asn
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Ala Lys Glu Gln Thr Thr Tyr Asp Met Ile Gln Lys Ser Phe His Phe
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Ala Leu Ser Leu Pro Asp Pro Met Tyr Lys Ser Asp Ser Trp Leu Gly
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Arg Gly Glu Pro Thr His Ile Ile Asp Tyr Leu Lys Phe Ser Ile Ala
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Arg Pro Ala Ile Asp Lys Glu Leu Glu Ala Phe His Asn Ala Met Lys
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gcg gcc aag gat aca gaa gac ggc gct cac ttt tgg gat ccg gat ctc   6055
Ala Ala Lys Asp Thr Glu Asp Gly Ala His Phe Trp Asp Pro Asp Leu
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gct tcc tac tac acg ttc ttc aag gag att agc gac aag tcg cga tcg   6103
Ala Ser Tyr Tyr Thr Phe Phe Lys Glu Ile Ser Asp Lys Ser Arg Ser
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tcc gca ctg cta ttc acg act ctg aag aac cgt atc ggc gaa gtc gag   6151
Ser Ala Leu Leu Phe Thr Thr Leu Lys Asn Arg Ile Gly Glu Val Glu
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aaa gaa tat ggc agg ttg gtc aaa aac aag gag atg aga gac agc aag   6199
Lys Glu Tyr Gly Arg Leu Val Lys Asn Lys Glu Met Arg Asp Ser Lys
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Asp Pro Tyr Pro Val Arg Val Asn Gln Val Tyr Glu Lys Trp Cys Ala
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atc acg cct gag gcg atg gac aaa tcc gga gca aat tat gat tct aag   6295
Ile Thr Pro Glu Ala Met Asp Lys Ser Gly Ala Asn Tyr Asp Ser Lys
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gtg atc agg ttg ctg gag ctg tcc ttc ctc gcg gac cgt gag atg aat   6343
Val Ile Arg Leu Leu Glu Leu Ser Phe Leu Ala Asp Arg Glu Met Asn
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Tyr Trp Arg Lys Asp Asp Ser Leu Asn Gln Ala Glu Ala Asn Phe Phe
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Asp Pro Asp Thr Leu Pro Trp Ser Lys Glu Pro Pro Arg Ala Ala Thr
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Tyr Pro Asp Pro Glu Val Tyr Glu Val Leu Gly Asp Asp Asp Phe Asp
1380 1385 1390
Gly Ile Gly Phe Thr Gly Asn Gly Asp Tyr
1395 1400

CLAIMS

1. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

2. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 1, wherein the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

3. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 2, wherein the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

4. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 3, wherein the domain is the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

5. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 4, wherein said nucleotide sequence encodes for a protein having the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

6. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain

according to claim 4, wherein said nucleotide sequence is the sequence of SEQ ID No. 1 or its complementary sequence.

7. Expression vector comprising, under the control of a promoter that is expressed in bacteria, the nucleotide sequence according to any one of claims 1-6.

8. Expression vector comprising, under the control of a promoter that is expressed in plants or in specific plant organs, the nucleotide sequence according to any one of claims 1-6 in a sense and anti-sense orientation.

9. Expression vector comprising, under the control of a promoter that is expressed in fungi, the nucleotide sequence according to any one of claims 1-6 in a sense and anti-sense orientation.

10. Expression vector comprising, under the control of a promoter that is expressed in animals, the nucleotide sequence according to any one of claims 1-6 in a sense and anti-sense orientation.

11. Prokaryotic organism transformed by using the expression vector active in bacteria according to claim 7.

12. Plants or a specific plant organ transformed by using the expression vector active in plants according to claim 8.

13. Plant mutated at the nucleotide sequence according to any one of claims 1-6 having a reduced or inhibited silencing activity.

14. Fungus transformed by using the expression vector active in fungi according to claim 9.

15. Fungus mutated at the nucleotide sequence according to any one of claims 1-6 having a reduced or inhibited silencing activity.

16. Non-human animal transformed by using the expression vector active in animals according to claim 10.

17. Non-human animal mutated at the nucleotide sequence according to any one of claims 1-6 having a reduced or inhibited silencing activity.

18. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain wherein the domain is at least 30 % homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

19. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 18, wherein the domain is at least 40 % homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1

20. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 19, wherein the domain is at least 50 % homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1

21. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 20, wherein the domain is the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

22. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 21 comprising the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

23. Use of the nucleotide sequence according to any one of claims 1-6 to modulate the gene silencing in plants, animals and fungi.

ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF

Abstract

A nucleotide sequence encoding for a protein characterized in that it has a silencing activity and comprises a RNA-dependent RNA polymerase domain is disclosed; furthermore expression vectors suitable for the expression of said sequence in bacteria, plants, animals and fungi are disclosed; the invention refers also to organisms transformed by such vectors.

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

MODULO DI DICHIARAZIONE PER DOMANDA DI BREVETTO

ITALIAN LANGUAGE DECLARATION

Io, sottoscritto inventore, dichiaro con il presente che:

As a below named inventor, I hereby declare that·

Il *mio domicilio*, recapito postale e cittadinanza sono quelli indicati in calce accanto al mio nome.

My residence, post office address and citizenship are as stated below next to my name

Che mi reputo in buona fede essere l'inventore originario, primo e unico (qualora un solo nominativo appaia elencato appresso) o il coinventore (qualora i nominativi siano più di uno) primo e originario dell'invenzione da me rivendicata, e per la quale faccio domanda di brevetto. Tale invenzione è chiamata:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed nd for which a patent is sought on the invention entitled:

Isolation and characterization of a *N. Crassa* silencing genes and uses thereof

E la sua descrizione è allegata alla presente *Dichiarazione a meno che non sia spuntata* la seguente casella:

the specification of which is attached hereto unless the following box is checked:

(**X** ..) il
è stata depositata una domanda di brevetto statunitense numero o una domanda di brevetto internazionale PCT numero
che è stata modificata il
(se del caso)

(x) was filed on **February 16, 2000**
as United States Application Number
or PCT International Application Number
PCT/IT00/00048
and was amended on
(if applicable)

Dichiaro inoltre con il presente di aver letto e compreso il contenuto della descrizione sopra indicata, comprese le rivendicazioni, come rettificata da qualsiasi emendamento a cui si sia accennato sopra.

Riconosco il mio dovere di rivelare informazioni che costituiscano materiale per l'esame della presente domanda secondo i termini del Titolo 37, Codice dei Regolamenti Federali, Comma 1,56(a)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1,56(a).

Italian Language Declaration

Con il presente rivendico i benefici di priorità per l'estero come stabilito dal Titolo 35, Codice degli Stati Uniti, Comma 119 per qualsiasi domanda di brevetto (o brevetti) straniera o per qualsiasi certificato di invenzione sotto elencato, ed ho anche elencato qui sotto tutte le domande di brevetto e certificati d'invenzione stranieri aventi una data di presentazione anteriore a quella della domanda per la quale si rivendica la precedenza:

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior foreign applications Domande all'estero precedenti			Priority claimed Priorità rivendicata			
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito				
RM99A000117	**IT**	**22.02.99**	(X) Yes	(..) No	(...) Yes	(. .) No
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito)	(...) Yes	(...) No	(...) Yes	(...) No
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito)	(...) Yes	(...) No	(...) Yes	(...) No

Con il presente rivendico il beneficio previsto dal Titolo 35, Codice degli Stati Uniti, Comma 120, per qualsiasi domanda (o domande) di brevetto sotto indicate, ed entro i limiti nei quali il materiale indicato in ciascuna delle domande di brevetto non è stato rivelato nella precedente domanda di brevetto americana nel modo previsto dal primo paragrafo del titolo 35, Codice degli Stati Uniti, Comma 112, riconosco il mio dovere di rivelare il materiale d'informazione, così come viene definito nel titolo 37, Codice dei Regolamenti Federali, Comma 1,56(a), che possa essere venuto ad aggiungersi nel periodo intercorso tra la data di presentazione della domanda precedente e la data nazionale o internazionale da PCT di presentazione di questa domanda:

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1,56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) Numero di domanda	(Filing Date) (Data di deposito)	(Stato Giuridico) (concessa, pendente, abbandonata)	(Legal Status) (patented, pending, abandoned)
(Application Serial No) Numero di domanda	(Filing Date) (Data di deposito)	(Stato Giuridico) (concessa, pendente, abbandonata)	(Legal Status) (patented, pending, abandoned)

Dichiaro inoltre con il presente che tutte le informazioni da me fornite sono per quanto mi consta vere e che tutte le affermazioni da me fatte sono per quanto mi consta vere; dichiaro inoltre che quando ho fatto queste affermazioni ero al corrente del fatto che false dichiarazioni fatte intenzionalmente sono punibili con multa o incarcerazione o ambedue, secondo quanto stabilito dalla sezione 1001 del Titolo 18 del Codice degli Stati Uniti e che tali informazioni intenzionalmente false possono mettere a repentaglio la validità della domanda di brevetto rilasciata in base ad esse.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Italian Language Declaration

PROCURA: Io, sottoscritto inventore, nomino con la presente il seguente Procuratore (o Procuratori) o Agente (o Agenti) che si incarica di perseguire questa pratica e di portare a termine tutte le operazioni necessarie all'Ufficio Brevetti pertinenti a questa pratica. (Elencare il Nome e il Numero di Matricola)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*List name and registration number)*

Recapito per la corrispondenza:

Send correspondence to:

Telefonare a: (Nome e Numero)

Direct telephone calls to: (name and telephone number).

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Fax.:

Nome completo dell'inventore primo o unico	Full name of sole or first\ inventor
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Firma dell'inventore Data **25.07.2001**	Inventor's signature Date
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Cittadinanza	Citizenship
Italian	
Recapito o Casella Postale	Post Office Address
Nome completo del secondo inventore, se esistente	Full name of second joint inventor, if applicable
Cogoni Carlo	
Firma dell'inventore Data: **25.07.2001**	Inventor's signature Date
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Residenza	Residence
Cittadinanza	Citizenship
Recapito o Casella Postale	Post Office Address

(Si prega di fornire le stesse informazioni e firme di eventuali terzi e più coinventori)

(Supply similar information and signature for third and subsequent joint inventors)